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Influence of Passive Ultrasonic Irrigation of the Photosensitizer Used in Photodynamic Therapy on Microbial Reduction in the Root Canal System: An *in Vitro* Study

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Introduction: The success of endodontic treatment is based on microbial reduction promoted by the interaction of chemical and mechanical procedures. Photodynamic therapy (PDT) is used as an adjunct to conventional treatment, with significant microbial reduction. This study aimed to evaluate the influence of passive ultrasonic irrigation (PUI) of the photosensitizer (PS) used in PDT on microbial reduction in the root canal system. **Materials and Methods**: Forty-five mesiobuccal root canals from extracted human mandibular molars that were inoculated with standard strains of *Enterococcus faecalis*, *Candida albicans*, and *Streptococcus mutans* for 21 days were selected. The root canals were prepared using the ProTaper Next system and randomly divided into 3 groups (n=15): 1) PDT: PDT alone; 2) PUI+PDT: PUI followed by PDT; and 3) PUI/PS+PDT: PUI of the PS followed by PDT. Microbiological samples were collected from the canals before and after using the protocols described above. The data were analyzed by analysis of variance (Tukey's test) at a significance level of 5%. **Results**: Microbial counts before PDT, PUI+PDT, and PUI/PS+PDT did not differ significantly (P>0.05), showing methodological standardization in the microbial contamination of the root canal system. There was a significant reduction in microbial counts after PDT (61.05%), PUI+PDT (65.04%), and PUI/PS+PDT (68.58%) (P<0.01), but with no statistically significant difference between the three groups (P>0.05). **Conclusion**: Based on the present findings, it was concluded that the combination of PUI with PDT or PS activation did not influence microbial reduction achieved by PDT alone.

Keywords: Endodontics; Methylene Blue; Photochemotherapy; Photosensitizing Agents; Root Canal Irrigants

Introduction

The anatomic complexity of the root canal system can lead to endodontic treatment failure by facilitating biofilm accumulation. Bacteria can penetrate up to 1000 µm into dentinal tubules, thus hindering the penetration of irrigants [1, 2].

Enterococcus (E.) faecalis, a facultative anaerobic microorganism, is highly resistant to conventional chemomechanical preparation [3] and is commonly found in persistent or secondary endodontic infections [4, 5]. It has the ability to form dense biofilms on the dentinal walls [5-7]. *Streptococcus (S.) mutans* is a highly prevalent microorganism in both asymptomatic and symptomatic endodontic infections [8] that has the ability to adhere and form biofilm on tooth surfaces and tolerate adverse environmental conditions [9]. Fungi are also found in infected root canals, with *Candida (C.) albicans* being the most commonly isolated fungus. It has the ability to adhere to the dentinal walls of root canals and to the surface of root canal filling materials [10].

Complementary techniques to conventional chemomechanical therapy have been investigated to tackle this bacterial resistance.

Passive ultrasonic irrigation (PUI) can improve root cleaning and disinfection efficacy and is considered an important auxiliary tool for cleaning the root canal system [11]. Other techniques have also been used to improve root disinfection, such as photodynamic therapy (PDT). Photodynamic therapy is based on the concept of cell death through the interaction of a light source and photosensitizers (PS) [12], and has been suggested as an effective adjunctive treatment strategy to eliminate endodontic pathogens [13, 14]. It is a minimally invasive treatment with no adverse effects on adjacent tissues [4, 15] that does not cause microbial resistance [16].

Photodynamic therapy is a two-step procedure: application of a PS, followed by irradiation of the sensitized tissue. The PS is applied to the tissue and activated by a light source. In the presence of oxygen, the light is absorbed by the PS. The energy that is transferred from the activated PS to the available oxygen results in the formation of singlet oxygen and free radicals, which are toxic oxygen species [7, 16]. The main light sources used in PDT are low-intensity laser and light-emitting diode [17-19]. This therapeutic modality does not increase the temperature, which prevents the degeneration of periradicular tissues [20].

Phenothiazine-based dyes, such as methylene blue and toluidine blue, are the most commonly used synthetic PS [21-23]. Methylene blue is highly effective at inactivating gram-positive and gram-negative endodontic bacteria through diode laser irradiation [24]. The effectiveness of PDT is reliant on the penetration of the PS on the microbial cell surface [7].

Several studies have yielded results that support the efficacy of PDT as an adjunct to conventional endodontic treatment in reducing bacterial viability inside the root canal [1, 6, 14, 16-18, 24-27]. However, there are no studies that have evaluated the influence of PUI of the PS used in PDT on microbial reduction in the root canal system, which was the objective of this study. The null hypothesis was that PUI of the PS would not produce a greater microbial reduction in the root canal system than PDT alone.

Materials and Methods

The present study was approved by the Ethics Committee of São Leopoldo Mandic Dental Research Center (CAAE number 21332919.7.0000.5374 and approval number 3.651.035). Forty-five freshly extracted permanent mandibular molars were selected based on the following inclusion criteria: fully formed apices and foramina; multi-rooted teeth with distinct mesiobuccal and mesiolingual canals; no prior endodontic treatment, no internal or external root resorption, no calcifications; no root carious lesions, dilacerations, or cracks; mesial canals with moderate root curvature of 10° to 20° [28]; and canals with initial anatomic diameter compatible with a size 10 K-file.

Specimen cleaning

Teeth were collected and stored in 0.1% thymol solution (Farmarim, Colatina, ES, Brazil), and their root surfaces were scraped with a size 14 periodontal curette (Hu-Friedy, Chicago, IL, USA), followed by prophylaxis with pumice (Asfer, São Caetano do Sul, SP, Brazil) and water.

Specimen standardization

All specimens were radiographed in the buccolingual direction. The crowns were sectioned using a silicon carbide disc (Carbodent; Gysi AS, Buenos Aires, Argentina) to standardize the root length at 15 mm. Verification of the root length measurement at 15 mm in the cervical-to-apical direction portion of the mesial root was made with a digital caliper (MTX, Xiamen, China). The distal root was sectioned, and the mesiolingual canal was sealed with Z250 XT composite resin (3M ESPE, Sumaré, SP, Brazil) [25]. A size 10 stainless steel K-file (Dentsply, Maillefer, Ballaigues, Switzerland) was inserted into the root canal until its tip was visible at the apical foramen. The working length was visually determined at 1 mm short of this measurement. The apical foramen was sealed with epoxy resin (Araldite, Embu das Artes, SP, Brazil), and the outer surface of the root was waterproofed with a layer of nail polish (Impala, Guarulhos, SP, Brazil). Prior to contamination, the root canals were prepared with a size 20 K-file (Dentsply, Maillefer, Ballaigues, Switzerland) up to the predetermined working length in order to increase the diameter for bacterial inoculation. All specimens were packaged and autoclaved at 121°C for 15 min.

Preparation of E. faecalis, C. albicans, and S. mutans suspension

Specimens were distributed in 24-well cell culture plates (Nest Biotechnology Co., Ltd., China). Standard strains of *E. faecalis* (ATCC 29212), *C. albicans* (ATCC 10231), and *S. mutans* (ATCC 25175) were reactivated in brain-heart infusion (BHI) broth (Difco, Detroit, MI, USA) and incubated at 37°C for 24 h in a 5% carbon dioxide (CO_2) atmosphere. The 24-h cultures were seeded onto a Petri dish (Nest Biotechnology Co., Ltd., Wuxi, Jiangsu, China) containing BHI agar (Difco, Detroit, MI, USA) and incubated at 37°C for 24 h in a 5% CO_2 atmosphere. After microbial growth, the culture suspension was prepared in a test tube containing 10 mL of sterile saline (0.9% sodium chloride) (Arboreto Ltda, Juíz de Fora, MG, Brazil) and matched to a 10 McFarland standard (Probac do Brasil Produtos Bacteriológicos, São Paulo, SP, Brazil). Then, in a sterilized test tube, 5 mL of the prepared suspension was mixed with 5 mL of BHI broth (Difco, Detroit, MI, USA) to obtain the final suspension.

Contamination of the specimens with E. faecalis, C. albicans, and S. mutans

A 20-µL aliquot of the final suspension was injected into the root canal using a 10-mL BD syringe (Becton Dickinson Indústria Cirúrgica, Curitiba, Paraná, Brazil) with a BD 20×0.55 24G hypodermic needle (Becton Dickinson Indústria Cirúrgica, Curitiba, PR, Brazil). A cotton pellet soaked in the microbial suspension was placed at the entrance of the root canal. Cotton pellets moistened with sterile distilled water were added to 4 wells of each cell culture plate (Nest Biotechnology, Wuxi, Jiangsu, China) to ensure a humid environment. The plate lid was closed and sealed, and the setup was incubated at 37°C for 21 days in a 5% CO_2 atmosphere. Every day, 20 µL of BHI broth (Difco, Detroit, MI, USA) was injected into the root canal using a 10-mL BD syringe (Becton Dickinson, Curitiba, PR, Brazil) with a BD 24G hypodermic needle (Becton Dickinson Indústria Cirúrgica, Curitiba, PR, Brazil).

Confirmation of contamination

The viability and purity of the microorganisms inside the root canal were checked weekly by random sampling of 2 specimens using a size 20 sterile paper point (Endopoints Industrial da Amazonia LTDA, Manacapuru, AM, Brazil). The paper point was kept inside the canal for 1 min, seeded on BHI agar plates (Difco, Detroit, MI, USA), and incubated at 37°C for 24 h in a 5% CO_2 atmosphere. After growth, smears and Gram stains were prepared for morphological and stain-based confirmation of the microorganisms.

Biomechanical preparation of root canals

All instrumentation and irrigation procedures were performed by the same operator, a specialist in endodontics with experience in the system used in this study.

All specimens were prepared using the same instrumentation protocol, with standardization of root canal diameter. Mesiobuccal root canals were prepared using the ProTaper Next rotary system (Dentsply Sirona, Ballaigues, Switzerland) driven by the X-Smart Plus motor (Dentsply, Maillefer, Ballaigues, Switzerland) at a speed of 300 rpm and a torque of 2 N. The X1 (size 0.17, taper 4%) and X2 (size 0.25, taper 6%) instruments were used in the working length, alternating with a size 10 K-file (Dentsply, Maillefer, Ballaigues, Switzerland), which was used to maintain apical patency.

During root canal preparation, the specimens were irrigated with 5 mL of saline (Arboreto, Juíz de Fora, MG, Brazil) at each instrument change or when one-third of the root was instrumented, for a total volume of 15 mL per root canal. Irrigation was performed using a 5-mL disposable hypodermic syringe (Injex, Ourinhos, SP, Brazil) and a NaviTip irrigation needle (Ultradent, Indaiatuba, SP, Brazil) with in-and-out movements. The needle was inserted into the canal 3 mm short of the working length.

Experimental groups

The teeth were randomly divided (by www.random.org) into 3 groups (n=15 each): PDT, PUI+PDT, and PUI/PS+PDT. The sample size was calculated with the results of the pilot study with 5 samples per group by analysis of variance for a minimum difference between the treatment medians of 0.12, an error deviation of 0.086, number of treatments of 6, power of 0.80, and alpha of 0.05. The number of samples per group calculated was 15.

PDT group

PDT was performed after completion of chemomechanical preparation. The root canal was filled with 2 mL of 0.01% methylene blue (Chimiolux, DMC, São Carlos, SP, Brazil) using a plastic syringe and needle. The PS was left inside the root canal for 5 min (pre-irradiation period) [15, 20, 29]. The canal was then irradiated with a 660-nm diode laser (MMOptics, São Carlos, SP, Brazil) using an intracanal optic fiber (MMOptics, São Carlos, SP, Brazil) of conical shape and 200 µm in diameter, attached to the tip of the device, with a power of 100 mW and energy of 9 J for 90 sec. The intracanal optic fiber was inserted into the canal 2 mm short of the working length [15, 20].

PUI+PDT group

Passive ultrasonic irrigation was performed after completion of chemomechanical preparation. An ultrasonic device (Gnatus, Barretos, SP, Brazil) and the E1-Irrisonic tip (Helse, Santa Rosa do Viterbo, SP, Brazil) were used at 20% power [30], with the diameter equivalent to a size 20 K-file and .01 taper. Ultrasonic activation was based on the protocols described by Van der Sluis *et al.* [31], 3 cycles of 20-sec saline activation, 3 cycles of 20-sec ethylenediaminetetraacetic acid activation, and 3 cycles of 20-sec saline activation. The E1-Irrisonic tip was inserted into the canal 2 mm short of the working length. Photodynamic therapy was performed as described for the PDT group.

PUI/PS+PDT group

Passive ultrasonic irrigation of the PS was performed after the completion of chemomechanical preparation. The root canal was filled with 2 mL of 0.01% methylene blue (Chimiolux, DMC, São Carlos, SP, Brazil) using a plastic syringe and needle. After the 5-min pre-irradiation period, the PS was agitated using an ultrasonic device (Gnatus, Barretos, SP, Brazil) at 20% power [30]. The E1-Irrisonic tip (Helse, Santa Rosa do Viterbo, SP, Brazil) was inserted into the canal 2 mm short of the working length, and the PS agitation sequence was performed for 1 min [20]. Photodynamic therapy was performed as described for the PDT group.

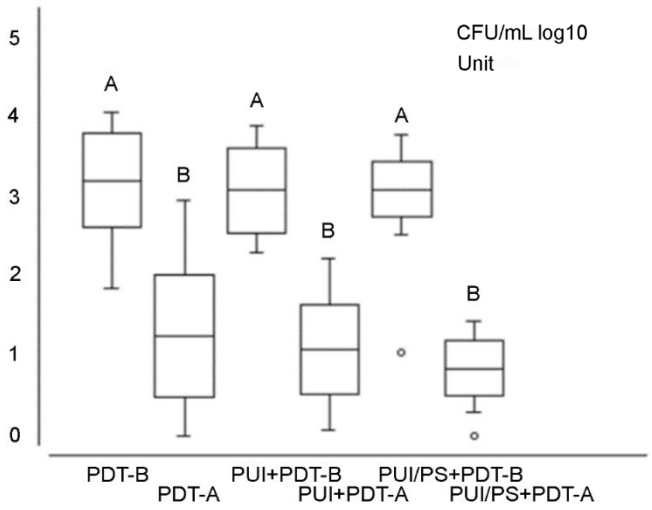

Figure 1. Box plot/standard deviations and analysis of variance (Tukey's test) statistical analysis of bacterial counts in the experimental groups (log_{10}). (Different uppercase letters indicate statistically significant differences)

Microbiological processing

Samples were collected before and after the disinfection protocols by inserting a size 25 sterile absorbent paper point (Endopoints Industrial da Amazonia LTDA, Manacapuru, AM, Brazil) into the root canal. The paper point was kept inside the canal for 1 min and transferred to a polypropylene tube (Eppendorf, Hamburg, Germany) containing 1 mL of sterile saline (0.9% sodium chloride) (Arboreto, Juíz de Fora, MG, Brazil). The samples were mixed for 30 sec on a vortex mixer (Vortex AD 56; Phoenix, Araraquara, SP, Brazil). Serial dilutions were prepared from this suspension until reaching a concentration of 10^4. A 0.1-mL aliquot of the suspension and of each dilution was cultured in Petri dishes (Nest Biotechnology, Wuxi, Jiangsu, China) containing BHI agar (Difco, Detroit, MI, USA). Seeded dishes were incubated at 37°C for 24 h in a 5% CO_2 atmosphere. The number of colony forming units (CFUs) per dish was counted, and the CFU/mL was calculated.

Statistical analysis

Statistical analysis was performed using Biostat 5.30 (AnalystSoft, Walnut, CA, USA). The results for CFU/mL were log10-transformed. The Lilliefors test was applied, and the data showed a normal distribution. The data were analyzed by analysis of variance and the intra-group statistical test was carried out with Tukey's test at a significance level of 5%.

Results

There was a significant reduction in microbial counts after PDT (61.05%), PUI+PDT (65.04%), and PUI/PS+PDT (68.58%) (P=0.008, P=0.004, and P=0.002, respectively, Table 1).

Microbial counts before PDT, PUI+PDT, and PUI/PS+PDT did not differ significantly (P=0.5122), showing methodological standardization in the microbial contamination of the root canal system in the different experimental groups (Figure 1).

Microbial reduction after PDT, PUI+PDT, and PUI/PS+PDT did not show statistically significant differences (p=0.5680). The combination of PUI with PDT or PS activation did not result in a significantly greater microbial reduction than that obtained with PDT alone (Figure 1).

Discussion

In endodontic infections, microorganisms penetrate the dentinal tubules and inhibit the action of conventional chemical and mechanical agents. Photodynamic therapy promotes disinfection of the root canal system and is a therapeutic alternative to further reduce microbial load achieved by instrumentation [6, 13, 14, 16-18, 21, 25-27]. However, to this day, no study has evaluated whether PUI of the PS during the pre-irradiation period influences microbial reduction in the root canal system, which was the objective of the present study.

Forty-five mesiobuccal root canals from mandibular molars were used in this study, which had been inoculated with *E. faecalis*, *S. mutans*, and *C. albicans* for 21 days according to Bumb *et al.* [1] and Pinheiro *et al.* [25]. The choice of molars in this work was to resemble the situation of greater clinical difficulty in penetration

Table 1. Arithmetic means (AM), standard deviations (SD) and analysis of variance (Tukey's test) statistical analysis of bacterial counts in the experimental groups (log_{10})

	PDT		PUI+PDT		PUI/PS+PDT	
	Before	**After**	**Before**	**After**	**Before**	**After**
AM (SD)	3.21 (0.59)[a]	1.25 (0.76)[b]	3.09 (0.53)[a]	1.08 (0.56)[b]	2.96 (0.63)[a]	0.93 (0.67)[b]
(p)	0.008		0.004		0.002	
%R	61.05%		65.04%		68.58%	

PDT, photodynamic therapy; PUI+PDT, passive ultrasonic irrigation and photodynamic therapy; PUI/PS+PDT, passive ultrasonic irrigation of the photosensitizer and photodynamic therapy; %R, percentage of microbial reduction; different letters indicate statistically significant differences

of the PS that occurs in the molars. Studies using single-rooted teeth present greater sample standardization; however, the ease of insertion and penetration of the PS is greater than in molars. As the objective of this study was to evaluate the influence of PUI on the PS, the choice of molars was made to be similar to the clinical condition with greater difficulty in PS penetration and the possible interference of PUI in this process.

The objective of contamination was to form a mature biofilm, thus favoring bacterial adhesion to the dentinal tubules. All root canals were enlarged prior to contamination to standardize their diameter for bacterial inoculation, in agreement with Pinheiro *et al.* [25]. The culture method for evaluating microbial reduction was chosen based on previous studies, which also used *ex vivo* samples [6, 14, 32-35].

The choice of methylene blue as the PS in this study was consistent with the studies conducted by Bumb *et al.* [1], Ghinzelli *et al.* [20], Pinheiro *et al.* [25], Soares *et al.* [36], and Asnaashari *et al.* [27]. The dye was used with a 5-min pre-irradiation period [14, 15, 18, 20, 29] to allow its penetration through the dentinal tubules and interaction with bacterial walls.

This study used an intracanal fiberoptic for transmitting the light, in agreement with Garcez *et al.* [37], Garcez & Hamblin [21], Souza *et al.* [15], Soares *et al.* [36], Moradi Eslami *et al.* [18], Asnaashari *et al.* [27], Ghorbanzadeh *et al.* [19], Ghorbanzadeh *et al.* [34], and Okamoto *et al.* [23]. This allowed a homogeneous distribution of laser light along the root canal up to the apical third [17].

Saline was used as the irrigating solution. This choice was due to the fact that chemical irrigants commonly used in endodontic therapy have antimicrobial effects and the objective of this study was to investigate the effect solely and exclusively of PUI and PDT, without additional effects of other chemical solutions that have disinfectant properties [15, 20, 25].

Passive ultrasonic irrigation was used to evaluate whether it would increase disinfection effectiveness in the root canal. In this study, PUI did not result in a significant difference in disinfection effectiveness compared with PDT alone; therefore, the null hypothesis was accepted. This suggests that both techniques were effective for microbial reduction, which is in agreement with Yang & Kim [35], who compared the antibacterial effectiveness of PUI and gallium laser and concluded that both were effective at reducing *E. faecalis* in single-rooted teeth and that the combined use of PUI and laser irradiation can enhance the antibacterial effect on dentinal tubules.

The present results showed a significant microbial reduction in all experimental groups after the disinfection protocols were performed, which is in agreement with Bumb *et al.* [1], Ghinzelli *et al.* [20], Hoedke *et al.* [6], and Batinic *et al.* [32]. According to Bumb *et al.* [1], PDT is effective at disinfecting deeper dentinal tubules in a root canal system at a depth of 890 to 900 μm. Similarly, our results also showed that PDT can significantly reduce bacterial biofilm formation in the root canal system. Similar results were also reported by Chiniforush *et al.* [13], Pinheiro et al. [25], Hoedke *et al.* [6], Sebrão *et al.* [4], Batinic *et al.* [32], Moradi Eslami *et al.* [18], Niavarzi *et al.* [33], Asnaashari *et al.* [27], and Karaoglu *et al.* [14].

The literature already presents papers that studied the effect and efficiency of different PDT protocols in endodontic treatments [38], including the removal of debris and smear layer [39] including open apex situations [40-42].

The groups using an ultrasonic agitation device (PUI+PDT and PUI/PS+PDT) showed results similar to those of the PDT group. Therefore, it can be assumed that both the active and passive use of a PS produce comparable microbial reduction in the root canal. Increased penetration of the PS with the use of PUI, as observed by Galler *et al.* [43], does not result in greater microbial reduction. Passive deep penetration of the PS is possibly compatible with the laser range, thus providing the best possible photodynamic effect. Further studies evaluating other PS, PUI parameters, and PDT are needed to investigate new clinical protocols that can further reduce microbial load achieved by PDT alone, with the purpose of obtaining the best possible disinfection effect in the root canal system.

Conclusion

Based on the present findings, it was concluded that the combination of PUI with PDT or PS activation did not influence microbial reduction achieved by PDT alone.

Acknowledgments

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Conflict of interest

None.

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Authors' contributions

Conception and design: DGPR; Analysis and interpretation: RAP; Data collection: CEF; Writing the article: DSSA; Critical revision of the article: ASM; Final approval of the article: CESB, SLP; Statistical analysis: SLP; Overall responsibility: DSSA

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